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Tomato TILLING Technology: Development of a Reverse Genetics Tool for the Efficient Isolation of Mutants from Micro-Tom Mutant Libraries

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To accelerate functional genomic research in tomato, we developed a Micro-Tom TILLING (Targeting Induced Local Lesions In Genomes) platform. DNA pools were constructed from 3,052 ethyl methanesulfonate (EMS) mutant lines treated with 0.5 or 1.0% EMS. The mutation frequency was calculated by screening 10 genes. The 0.5% EMS population had a mild mutation frequency of one mutation per 1,710 kb, whereas the 1.0% EMS population had a frequency of one mutation per 737 kb, a frequency suitable for producing an allelic series of mutations in the target genes. The overall mutation frequency was one mutation per 1,237 kb, which affected an average of three alleles per kilobase screened. To assess whether a Micro-Tom TILLING platform could be used for efficient mutant isolation, six ethylene receptor genes in tomato (*SlETR1–SlETR6*) were screened. Two allelic mutants of *SlETR1* (*Sletr1-1* and *Sletr1-2*) that resulted in reduced ethylene responses were identified, indicating that our Micro-Tom TILLING platform provides a powerful tool for the rapid detection of mutations in an EMS mutant library. This work provides a practical and publicly accessible tool for the study of fruit biology and for obtaining novel genetic material that can be used to improve important agronomic traits in tomato.

Keywords: Ethylene receptor • Fruit ripening • Micro-Tom • Reverse genetics • TILLING • Tomato.

Abbreviations: CAPS, cleaved amplified polymorphic sequence; CODDLE, codons optimized to discover deleterious lesions; DAF, days after flowering; EMS, ethyl methanesulfonate; EST, expressed sequence tag; GABA, γ-aminobutyric acid; NBRP, National BioResource Project; SOL, The International Solanaceae Genomics Project; TILLING, Targeting Induced Local Lesions In Genomes.

Introduction

Tomato (*Solanum lycopersicum*) is a vegetable crop that is commercially valuable worldwide. It is a model system for studying many aspects of fruit biology, including development, ripening and metabolism (Giovannoni 2004, Tanksley 2004, Carrari and Fernie 2006, Giovannoni 2007, Barry and Giovannoni 2007, Pineda et al. 2010). Insight into the carotenoid biosynthetic pathway, chromoplast differentiation (Isaacson et al. 2002, Galpaz et al. 2008, Egea et al. 2010, Stigliani et al. 2011) and γ-aminobutyric acid (GABA) metabolism (Akihiro et al. 2008, Yin et al. 2010b) has been obtained using tomato as a model plant. Tomato has been widely used for studies of the biotic and abiotic response, such as salinity and wounding stress (Yin et al. 2010a, Sun et al. 2010, Sato et al. 2011) and infection by plant-parasitic nematodes (Bhattarai et al. 2008, Uehara et al. 2010). Large-scale expressed sequence tag (EST) analysis demonstrated that approximately 30% of expressed genes in tomato had no significant similarity to Arabidopsis genes (Van der Hoeven et al. 2002). Tomato is a suitable system for studying unique biological phenomena not harbored by Arabidopsis.

The tomato genome was entirely sequenced by The International Solanaceae Genomics Project (SOL), and many of the gene sequences can be retrieved from databases (Mueller et al. 2005a, Mueller et al. 2005b, Mueller et al. 2009). Now, it is important to use the genome sequence information to elucidate the gene functions. TILLING (Targeting Induced Local Lesions In Genomes) allows for the identification of an allelic series of mutants with a range of modified functions for desired genes (McCallum et al. 2000a, McCallum et al. 2000b, Colbert et al. 2001, Comai and Henikoff 2006). TILLING has been applied in many model and crop species, with no exception of tomato. In contrast to transgenic methods,

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mutagenesis has the advantage of producing mutant alleles directly for use in breeding programs as genetic sources or markers. Ethyl methanesulfonate (EMS) and fast-neutron mutant populations have been created in the M82 genetic background. Mutants were classified according to their visible phenotype, and this information is publicly available through a database (Menda et al. 2004). Using this M82 EMS mutant population, a TILLING platform was recently created by another group (Piron et al. 2010). Other cultivars, such as cv. Red Setter and cv. TPAADASU (Gady et al. 2009, Minoia et al. 2010), also served as material for creating EMS populations, and TILLING platforms were developed from these mutant populations.

Recently, a dwarf tomato cv. Micro-Tom has been extensively used as a model variety to accelerate functional genomic research (Iijima et al. 2008, Aoki et al. 2010). Like Arabidopsis, Micro-Tom is a suitable model plant due to its small plant size and short life cycle, and the existence of high efficiency transformation methods and indoor cultivation protocols under normal fluorescent conditions (Meissner et al. 1997, Emmanuel and Levy 2002, Sun et al. 2006, Watanabe et al. 2007). To establish functional genomic tools in tomato, we previously developed nearly saturated Micro-Tom EMS and γ-ray-irradiated mutant populations (Matsukura et al. 2007, Watanabe et al. 2007, Saito et al. 2011). Information on this mutant collection is available in the TOMATOMA database (http://tomatoma.nbrp.jp/). Although numerous tomato mutant populations have been generated by several groups, reports on large-scale mutant screening are limited, probably due to the large size of common tomato cultivars used in those studies. The cultivation of common cultivars requires extensive space in the greenhouse or field, making it difficult for most researchers to characterize even a single mutant. Therefore, a TILLING platform made from the Micro-Tom mutant population would greatly facilitate the functional analysis of tomato genes.

Several fruit-ripening mutants have been reported in tomato, such as *ripening-inhibitor* (*rin*), *Colorless non-ripening* (*Cnr*), *non-ripening* (*nor*), *Green-ripe* (*Gr*) and *Never-ripe* (*Nr*). The causative genes were isolated and shown to act as upstream regulators of the ethylene signaling network or in ethylene perception (Wilkinson et al. 1995, Vrebalov et al. 2002, Giovannoni 2004, Barry et al. 2005, Manning et al. 2006, Barry and Giovannoni 2007). These previously characterized mutants were mostly selected from a production field or a breeding program as spontaneous variants (Giovannoni 2007). Tomato has six ethylene receptor genes in its genome (Klee 2004). Ethylene receptors control the fruit ripening process via the perception of ethylene. *Nr* is a well-characterized ethylene receptor mutant that exhibits a strong ethylene-insensitive phenotype and inhibition of fruit ripening (Lanahan et al. 1994, Yen et al. 1995). No new ethylene receptor mutant has been isolated in the past decade from tomato.

In this study, the Micro-Tom TILLING platform was evaluated as a reverse genetics tool for the isolation of novel ethylene receptor mutant alleles. To attain this objective, six ethylene receptor genes (*SlETR1*–*SlETR6*) were screened by TILLING in our Micro-Tom mutant population. As a result, two *Sletr1* mutant alleles (*Sletr1-1* and *Sletr1-2*) with reduced ethylene responses were identified. Delayed fruit ripening and prolonged fruit shelf life were observed in these mutants. We report that the Micro-Tom TILLING platform serves as a powerful tool for studying fruit biology and accelerates the isolation of important agronomic traits in tomato. The use of this tool as a new methodology is discussed.

Results

Mutation screening of a Micro-Tom EMS mutant collection by TILLING

To apply TILLING methodology to the Micro-Tom mutant collection, DNA samples were prepared from 3,052 EMS-mutagenized M_2 lines, generated in the presence of either 0.5% EMS (2,180 lines) or 1.0% EMS (872 lines) (Watanabe et al. 2007, Saito et al. 2011) (**Fig. 1**). DNA superpools consisting of eight families per pool were subjected to a first screening. We used the CODDLE (Codons Optimized to Discover Deleterious Lesions) program for primer design to select optimal target amplicons, as performed previously (Dahmani-Mardas et al. 2010, Piron et al. 2010). All steps of the mutation screening were carried out by the procedure shown in **Figs 1** and **2**. To estimate the mutation frequency of the Micro-Tom mutant collection and to assess its utility in the study of fruit biology, we screened for mutations in 10 genes involved in fruit ripening, softening and γ-aminobutyric acid (GABA) metabolism. A total region of the tomato genome of approximately 15.3 kb was screened and 35 mutations were identified, among which 18 were from the 0.5% EMS population and 17 were from the 1.0% population (**Table 1**). Tansition from G/C to A/T was observed in 91.4% of the mutations, known as the major nucleotide substitution induced by EMS (Greene et al. 2003), while non-G/C to A/T substitution was also observed at low frequency (**Supplementary Table S2**). The mutation frequency was calculated as follows: the size in base pairs of the region screened was multiplied by the total number of lines screened per total number of identified mutations. The 0.5% EMS population had a mild mutation frequency of one mutation per 1,710 kb, whereas the frequency of the 1.0% EMS population was one mutation per 737 kb (**Table 1**). One to six mutated alleles were obtained per screened gene. Among the exonic mutations, the ratio of silent and missense mutations was 16.0 and 80.0%, respectively, and the ratio of nonsense mutations causing a stop codon was 4.0% (**Table 2**).

Identification of novel *Sletr1* mutant alleles caused by EMS mutagenesis

Mutations in the transmembrane region of ethylene receptor genes are known to affect the ethylene sensitivity in both Arabidopsis and tomato dramatically (Chang et al. 1993,

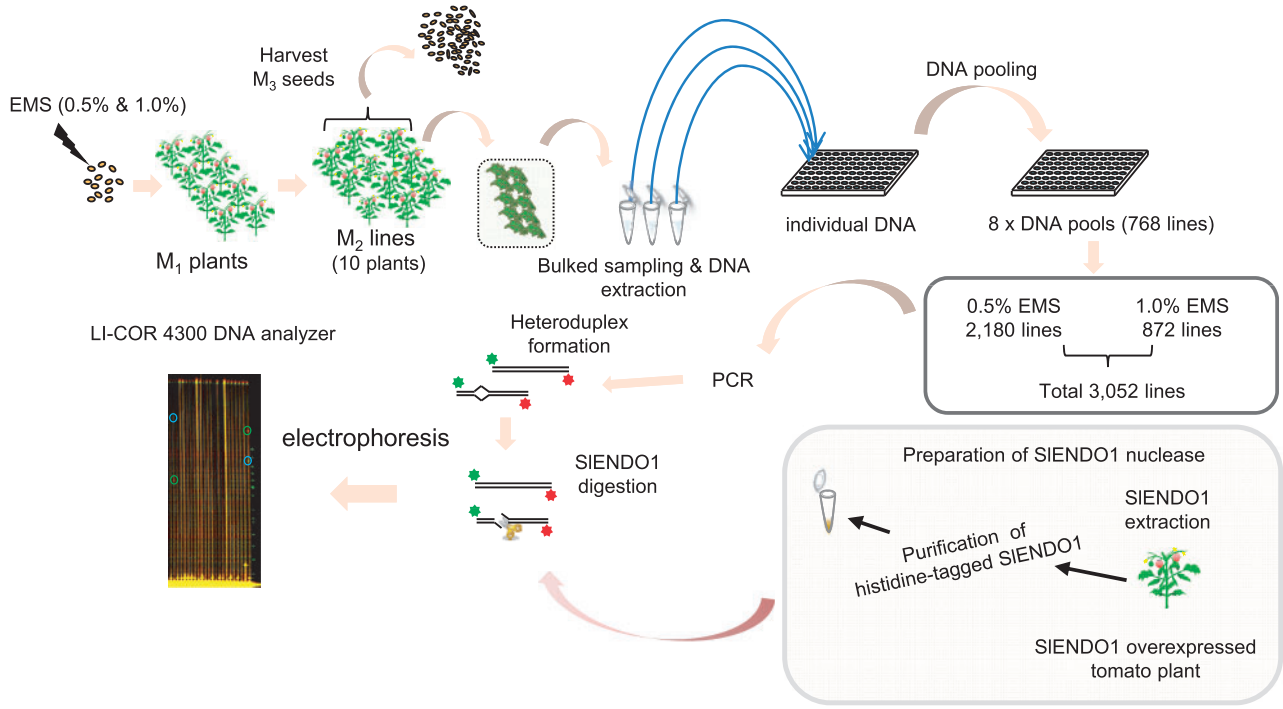

Fig. 1 Outline of Micro-Tom TILLING platform. Seeds were mutagenized with 0.5 or 1.0% EMS. To avoid ambiguities caused by chimerism of mutant plants in the M_1 generation, plants were self-fertilized. The M_2 progeny of 10 seeds per line was used for screening. The DNA pool and M_3 seeds were collected from each M_2 line (10 plants/line). An 8× DNA pool was used for the screening. PCR was performed using 5′ end-labeled gene-specific primers to target the desired region, and heteroduplexes were formed by heating and cooling the PCR products. Recombinant SlENDO1 nuclease was used to cleave base mismatches, and products that represent induced mutations were visualized by denaturing PAGE. Recombinant SlENDO1 was extracted and purified from SlENDO1-overexpressing transgenic tomato plants.

Wilkinson et al. 1995). Therefore, by means of TILLING, we expect to screen effectively for and isolate various novel mutant alleles of ethylene receptor genes with weak and strong phenotypes and even for those with loss of function. We performed TILLING screening of six ethylene receptor genes in tomato, *SlETR1*–*SlETR6*, and found more than one allele for each gene (**Table 1**). A possible loss-of function mutant caused by a nonsense mutation (Q368stop) was found for *SlETR5* (**Supplementary Table S1**). Two *ETR1* mutant alleles (*Sletr1-1* and *Sletr1-2*) were found; each had an amino acid substitution in the predicted transmembrane region, c152t (P51L) for *Sletr1-1* and t206a (V69D) for *Sletr1-2* (**Fig. 3**). The P51L substitution in *Sletr1-1* corresponds to the same mutation position as *Never ripe*, where P36 was substituted to L in *Nr* (Wilkinson et al. 1995) (**Supplementary Fig. S2**), suggesting that the *Sletr1-1* mutation also affects receptor function and ethylene sensitivity. The hydrophobicity of the transmembrane region and the charge distribution in the flanking regions appear to be important determinants of the membrane orientation (Harley et al. 1998). The V69D substitution in *Sletr1-2* was located in the transmembrane-flanking region, as shown in the topological model of CmERS1 proposed by Ma et al. (2006). The average hydrophobicity of *Sletr1-2* decreased from 0.176 in the wild-type protein to 0.166, as calculated by the SOSUI program (http://bp.nuap.nagoya-u.ac.jp/sosui/) (Hirokawa et al. 1998), suggesting that this second allele of V69D would also affect the receptor function. Thus, we identified two *Sletr1* mutant alleles that possibly exhibited altered protein function.

Sletr1 mutant alleles resulted in ethylene insensitivity at different levels

In this study, the ethylene-related phenotypes of the *Sletr1* mutant alleles (*Sletr1-1* and *Sletr1-2*) were further characterized. We tested whether the M_3 generation of the mutant exhibited the triple response to exogenously applied ethylene. Plants carrying the mutant alleles exhibited the ethylene-insensitive phenotype, whereas wild-type seedlings became swollen, and hypocotyl and root elongation were inhibited in response to ethylene treatment (**Fig. 4A**). Similar to Arabidopsis *etr1-1*, *Sletr1-1* seedlings were completely ethylene insensitive (Chang et al. 1993, Hua et al. 1995), and failed to exhibit a reduction in hypocotyl and root length or an apical hook in response to ethylene treatment. The length of hypocotyls and roots were 58.7 ± 1.9 and 53.3 ± 4.7 mm in the absence of ethylene and 57.7 ± 0.5 and 56.0 ± 2.8 mm in the presence of ethylene (mean ± SD), respectively. *Sletr1-2* displayed a relatively weak ethylene insensitivity, and exhibited an intermediate level of hypocotyl length reduction compared with the wild type and *Sletr1-1*, from 50.7 ± 2.5 to 31.7 ± 2.4 mm in the *Sletr1-2*

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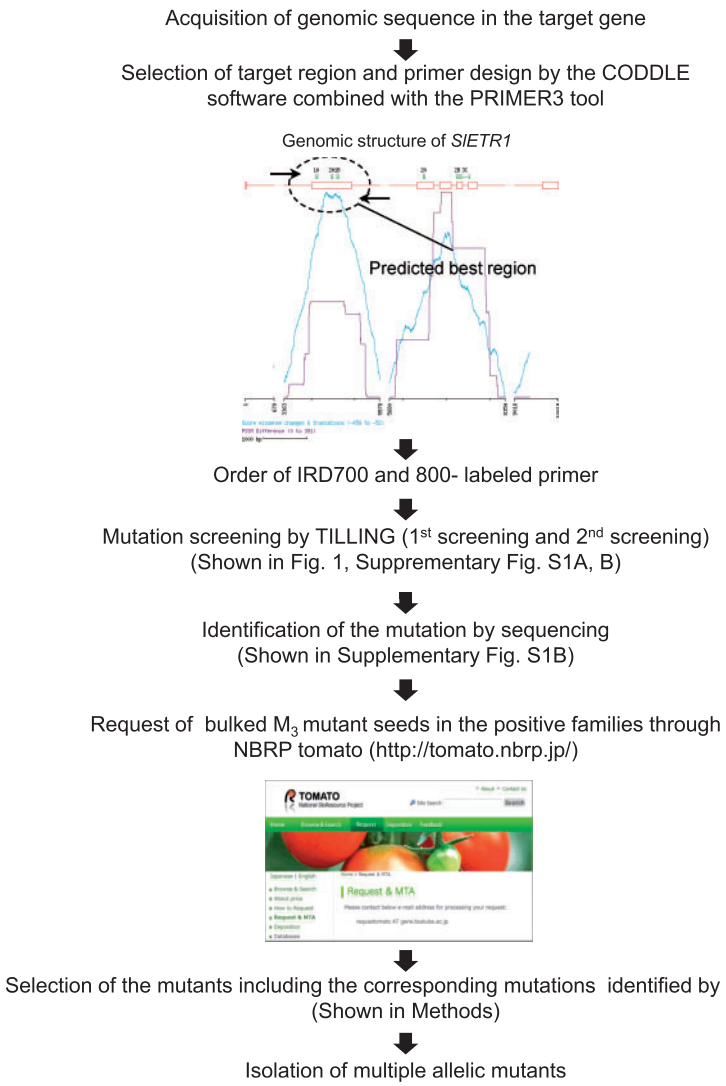

Fig. 2 Work flow of TILLING in this study. TILLING was performed in this study as described in the figure. See **Supplementary Fig. S1** for an image of the results of TILLING screening.

Table 1 Mutation frequency in the Micro-Tom EMS mutant population

Gene	Screened size (bp)	No. of screened M_2 lines		No. of identified mutations[a]		Mutation frequency[b]	
		0.5 % EMS	1.0 % EMS	0.5 % EMS	1.0 % EMS	0.5 % EMS	1.0 % EMS
SlETR1	1,403	2,180	872	4	2	1/765 kb	1/612 kb
SlETR2	1,185	2,180	872	2	0	1/1,292 kb	–
SlETR3 (*NR*)	945	2,180	872	0	2	–	1/412 kb
SlETR4	1,466	2,180	872	0	1	–	1/1,278 kb
SlETR5	1,447	2,180	872	1	4	1/3,154 kb	1/315 kb
SlETR6	1,456	2,180	872	0	1	–	1/1,269 kb
SlSSADH	1,572	2,180	872	1	2	1/3,427 kb	1/685 kb
SlGABAT1	3,537	2,180	872	7	4	1/1,102 kb	1/771 kb
SlGABAT3	1,007	1,516	–	2	–	1/763 kb	–
SlPL	1,348	664	872	1	1	1/865 kb	1/1,175 kb
Total/mean	15,366			18	17	1/1,710 kb	1/737 kb

[a] THe number of identified mutation is shown based on TILLING gel screening.
[b] The mutation frequency is calculated as follows: (size in base pairs of the screened region)×(total number of lines screened)/(total number of identified mutations).

seedlings. The reduction of root length in *Sletr1-2* was similar to that in *Sletr1-1* in the presence of 10 p.p.m. ethylene, showing only a slight reduction, from 57.3 ± 2.1 to 46.3 ± 2.4 mm in the *Sletr1-2* roots (Fig. 4A, B). The epinastic response exhibited a similar trend to the triple response. In the presence of 50 p.p.m. ethylene, the petiole exhibited downward curvature in the wild type, but showed a weaker or no response in *Sletr1-1* and *Sletr1-2* (Fig. 4C).

Sletr1 mutant alleles exhibited dominant inheritance

Constitutive overexpression of mutated ethylene receptor genes and genetic analyses in Arabidopsis and tomato have revealed that ethylene receptor mutants inherit the ethylene-insensitive phenotype in a dominant-negative manner (Chang et al. 1993, Lanahan et al. 1994, Wilkinson et al. 1995). To investigate the inheritance pattern of the obtained *Sletr1* mutants, we crossed the two mutants with wild-type Micro-Tom and Ailsa Craig. *Sletr1-1* and *Sletr1-2* F_2 seedlings were treated with 10 or 5 p.p.m. ethylene. The triple response was assayed for each BC_1S_1 population (*Sletr1-1*×Micro-Tom BC_1S_1, *Sletr1-2*×Micro-Tom BC_1S_1) and the result was scored as ethylene sensitive (5), intermediate (9) or insensitive (6) for the *Sletr1-1* BC_1S_1 population ($\chi^2 = 0.3$, $P > 0.8$), and ethylene sensitive (10), intermediate (18) or insensitive (10) for the *Sletr1-2* BC_1S_1 population ($\chi^2 = 0.237$, $P > 0.8$). The F_2 population (*Sletr1-1*×Ailsa Craig F_2, *Sletr1-2*×Ailsa Craig F_2) was scored as ethylene sensitive (10) or insensitive (33) for the *Sletr1-1* F_2 population ($\chi^2 = 0.07$, $P > 0.75$), and ethylene sensitive (26) or insensitive (63) for the *Sletr1-2* F_2 population ($\chi^2 = 0.843$, $P > 0.3$) (Table 3). The F_2 populations were scored only as ethylene sensitive and insensitive, because they exhibited an indistinguishable intermediate phenotype of different seedling lengths due to the effect of the Micro-Tom or Ailsa Craig genetic background. The segregation ratio of both of the BC_1S_1 and F_2 populations in *Sletr1-1* and *Sletr1-2* was nearly 1 : 2 : 1 in the BC_1S_1 and 3 : 1 in the F_2, respectively, suggesting that the progeny inherited both alleles as a single dominant trait. The inheritance pattern of the *Sletr1* mutant alleles was consistent with a previous report of the ethylene receptor mutant *Nr*, which displayed a semi-dominant triple response phenotype (Lanahan et al. 1994).

Table 2 The type of amino acid substitution in the exonic region

Effect	No. of mutations	Percentage (%)
Silent mutation	4	16.0
Missense mutation	20	80.0
Nonsense mutation	1	4.0
Total	25	100

Sletr1 mutant alleles resulted in delayed petal abscission and fruit ripening

Both *Sletr1* mutants showed delayed petal abscission (Fig. 5B). This phenotype is similar to that of the *Nr* mutant and RNAi

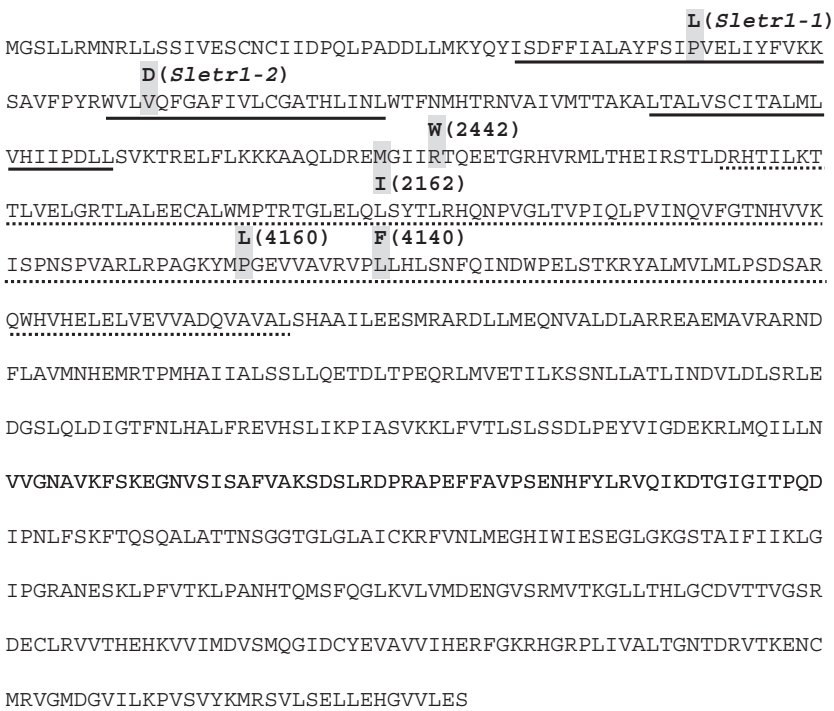

Fig. 3 The amino acid sequence of the tomato ethylene receptor (*SlETR1*) and sequence variants identified by TILLING. Vertical lines and a dotted line indicate the transmembrane region and GAF domain, respectively.

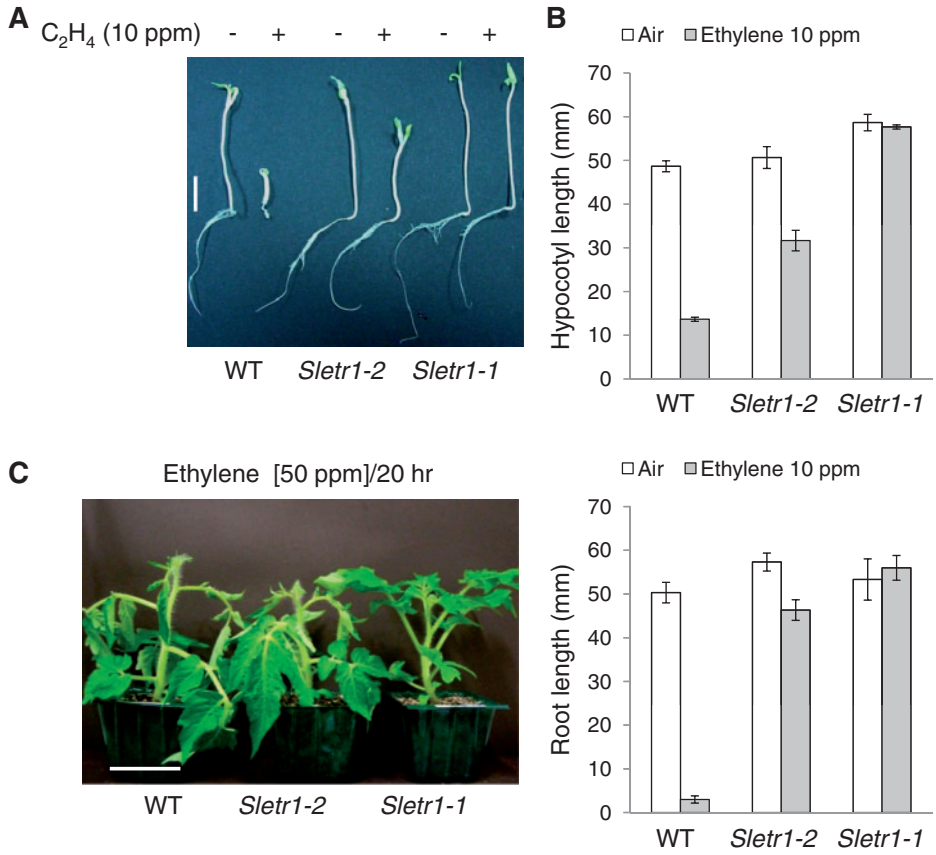

Fig. 4 The ethylene response of *Sletr1* mutant alleles. (A) Triple response phenotype of the wild type (WT), *Sletr1-2* and *Sletr1-1* seedlings. Seeds were surface sterilized and sown on 1/2 MS medium in the absence (−) or presence (+) of 10 p.p.m. C_2H_4 and incubated at 25°C in the dark for 7 d. Bar = 1 cm. (B) Quantification of ethylene-induced inhibition of root and hypocotyl growth. Vertical bars represent the SD ($n = 3$). (C) Epinastic response phenotype of 4-week-old wild-type (WT), *Sletr1-2* and *Sletr1-1* plants. Plants were treated with 50 p.p.m. ethylene at 25°C for 20 h. Bar = 5 cm.

Table 3 Inheritance pattern of *Sletr1* mutant alleles

Population[a]	F_2 and BC_1S_1 segregation[b] (mutant : WT)	χ^2 value[b]	Inheritance pattern[d]
Sletr1-1×Ailsa Craig F_2	33 : 10	0.07	Single dominant
Sletr1-1×Micro-Tom BC_1S_1	15 : 5	0	Single dominant
Sletr1-2×Ailsa Craig F_2	63 : 26	0.843	Single dominant
Sletr1-2×Micro-Tom BC_1S_1	28 : 10	0.035	Single dominant

[a] *Sletr1* mutant alleles were crossed with wild-type (WT) Micro-Tom and Ailsa Craig.
[b] The number of progeny exhibiting WT (ethylene-sensitive) or mutant (ethylene-insensitive) phenotypes in the F_2 and BC_1S_1 populations is shown. The number of progeny exhibiting intermediate ethylene insensitivity in BC_1S_1 is shown in the text.
[c] The χ^2 values were calculated for the F_2 and BC_1S_1 populations.
[d] Inheritance pattern was estimated based on the χ^2 value. The values were significant at the level of 5%.

(RNA interference) transgenic lines of tomato *ETHYLENE INSENSITIVE LIKE* (*EIL*) (Lanahan et al. 1994, Yokotani et al. 2009), which are associated with inhibition of the ethylene signaling pathway. Flowers with fully opened petals were defined as entering anthesis and tagged as 0 days after flowering (DAF). Wild-type Micro-Tom flowers began to wilt at 3 DAF, whereas flowers of *Sletr1* mutants did not wilt even at 15 DAF, except for flowers of heterozygous *Sletr1-2* which wilted at 5 DAF (**Fig. 5B**). Compared with *Sletr1-2*, *Sletr1-1* exhibited a more severe delayed abscission phenotype, and petals were still attached to the fruits even at 60 DAF (**Fig. 5A**). Delayed fruit ripening was observed in *Sletr1-1*, and the fruit turned fully yellow or orange 7–10 d later than did the wild type (**Fig. 5A**). At 60 DAF, most *Sletr1-1* fruits did not turn red, while some changed to an intermediate color between orange and red (**Supplementary Fig. S4**). No difference was observed between *Sletr1-2* and the wild type regarding the ripening phenotype (**Fig. 5A**). These results indicate that the degree of petal abscission and fruit ripening were associated with the level of ethylene sensitivity.

To confirm the linkage between the mutant alleles and phenotypes, we evaluated the abscission and ripening phenotype of each BC_1S_1 population. Genotyping of *Sletr1* mutant alleles was carried out by cleaved amplified polymorphic sequence (CAPS) analysis (see the Materials and Methods).

Petal abscission and the ripening phenotype in homozygous and heterozygous plants were compared with those in the wild type. Heterozygous plants exhibited an intermediate phenotype between wild-type and homozygous plants. Petals of heterozygous *Sletr1-2* plants began to wilt at 5 DAF, and almost separated from fruits at 7 DAF and 10 DAF (**Fig. 5B**). At the late ripening stage (50–60 DAF), heterozygous *Sletr1-1* fruits became red (**Fig. 5C**), indicating that the petal abscission and ripening phenotypes were semi-dominant in mature plants. The *Sletr1-1* and *Sletr1-2* mutations co-segregated with delayed petal abscission and ripening in each F_2 population (data not shown).

Fruit shelf life was prolonged in *Sletr1-2*

Fruit shelf life is an important property in determining the market value of tomato. We hypothesized that weak ethylene insensitivity might increase fruit shelf life, without visibly affecting the ripening color. The *Sletr1-2* mutant was an ideal plant to test this hypothesis. Wild-type and *Sletr1-2* fruits of the same stage (45 DAF) were harvested and stored in a sealed chamber at 25°C for 60 d. In wild-type fruits, black spots began to arise on the fruit surface at 20–25 d after harvest, whereas the *Sletr1-2* fruits did not show any spots (data not shown). The wild-type fruits deteriorated 60 d after harvest, whereas in the *Sletr1-2* fruits the surface remained intact (**Fig. 6**). This observation shows that fruit shelf life is dramatically prolonged in *Sletr1-2* and that deterioration of post-harvest fruit is delayed.

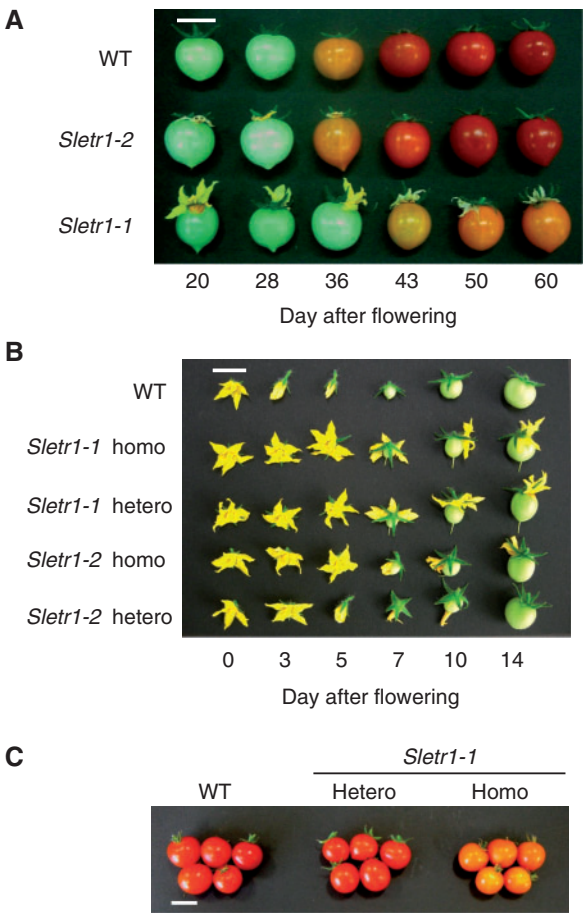

Fig. 5 Fruit ripening and petal abscission phenotype of *Sletr1* mutant alleles. Comparison of fruit ripening in the wild type (WT) and *Sletr1* mutant alleles. Fruits were harvested at different maturities. (B) Petal abscission phenotype in homozygous and heterozygous *Sletr1* mutants. (C) Fruit ripening phenotype in the homozygous and heterozygous *Sletr1-1* mutant. Bar = 2 cm. Homozygous and heterozygous plants were selected by CAPS analysis (shown in the Materials and Methods).

Discussion

The dwarf cultivar Micro-Tom has recently been identified as a model tomato (Matsukura et al. 2008). Genome information of Micro-Tom, such as full-length cDNA and bacterial artificial chromosome (BAC) sequences, has been accumulated by the Kazusa DNA Research Institute as part of the National BioResource Project (NBRP, MEXT) (Aoki et al. 2010). We previously developed a Micro-Tom mutant population and a database named 'TOMATOMA', which provides information about visible mutant phenotypes.

In this study, we generated a Micro-Tom TILLING platform from 3,052 EMS-mutagenized lines. We also deployed the LI-COR/ENDO1 system for detection of mutations, as a low-cost and high-sensitivity method (**Fig. 1**) (Triques et al. 2007, Triques et al. 2008, Piron et al. 2010). To be cost-effective, we extracted endonuclease I for digesting mismatch DNA from SlENDO1-overexpressing transgenic tomato plants (see the Materials and Methods). The mismatch DNA recognition and cleavage activity of SlENDO1 was evaluated by using different combinations of artificially designed mismatches. We could see

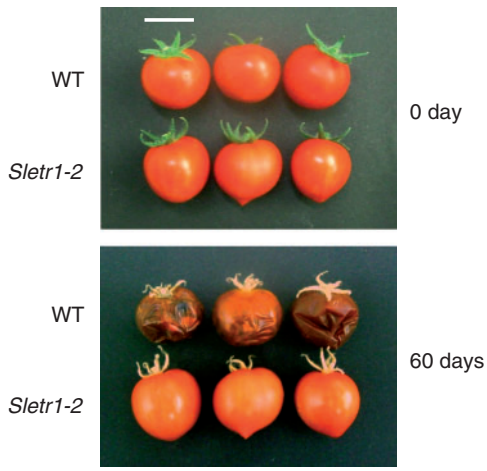

Fig. 6 Fruit shelf life of the *Sletr1-2* mutant. Fruits were harvested 45 d after flowering and stored at 25°C for 60 d in a sealed chamber. The appearance of *Sletr1-2* fruits was compared with that of the wild type at 60 days after harvest. Bar = 2 cm.

no bias in DNA mismatch types recognized and cleaved by SlENDO1 (**Supplementary Fig. S5A**), indicating a similar tendency to the Arabidopsis ENDO1 (Triques et al. 2007). The transgenic tomatoes expressing SlENDO1 stably provided the enzyme with a simple extraction procedure; we used this system in the TILLING analyses. Screening of 10 genes by targeting an approximately 15.3 kb region of the tomato genome has enabled us to estimate the mutation frequency to be one mutation per 1,710 kb for the 0.5% EMS population, and one mutation per 737 kb for the 1.0% EMS population. A correlation was observed between the concentration of EMS applied and the mutation frequency obtained, where the frequency was 2.32 times higher in the 1.0% population than in the 0.5% population. This result is consistent with the previously reported Red Setter mutant population (Minoia et al. 2010). The mutation frequency obtained for our 1.0% EMS population was lower than that of the Red Setter population (one per 322 kb; Minoia et al. 2010), but was similar to that of the TPAADASU population (one per 737 kb; Gady et al. 2009) and the M82 population (one per 574 kb; Piron et al. 2010). Thus, we concluded that the 1.0% populations are more efficient for screening mutants by applying TILLING using our platform. We intend to increase the size of the 1.0% population, and thereby increase the chance of isolating mutants of target genes.

Given that one would expect a higher concentration of EMS to result in a higher mutation frequency and to facilitate the identification of desired mutant alleles even in a small-scale population, more backcrossing might be necessary to characterize the specific mutant phenotypes due to the presence of mutations that might affect unpredictable plant growth. In fact, the Micro-Tom mutants recovered from the populations with higher EMS concentrations tend to show stronger infertility, most probably due to the multiple mutations that could affect reproductive development (Saito et al. 2011). Thus we suggest that the mutants isolated from our TILLING platform should be subject to backcrossing several times to remove undesirable mutations before mutant analysis.

Recently, a practical approach using identified mutants has been made by taking advantage of the TILLING platforms developed in many crop species. For example, interesting mutants were identified in tomato, namely the *SleIF4E1* G1485A mutant, which showed potyvirus resistance, and in melon, namely the *CmACO1* G194D mutant, which produced fruit with an enhanced shelf life (Dahmani-Mardas et al. 2010, Piron et al. 2010). These reports indicated that TILLING is a useful and could be a practical tool for crop improvement. Our study also provided evidence that the Micro-Tom TILLING platform can be used as a valuable tool for studying fruit biology and isolating genetic material that harbors important agronomic traits in tomato. Currently, >2,000 new lines have been added to the Micro-Tom TILLING population to improve the quality of the platform. This population will become available in the near future and will improve the possibility of identifying a greater number of interesting mutants.

As a result of *SlETR1* screening, we identified two mutant alleles (*Sletr1-1* and *Sletr1-2*) that exhibited reduced ethylene sensitivity (**Fig. 4**). These mutations were located in different domains of the transmembrane region, i.e. P51L (*Sletr1-1*) was in the first and V69D (*Sletr1-2*) in the second domain (**Fig. 3**). The P51L substitution of *Sletr1-1* corresponds to the same amino acid substitution of P36L in *Nr* and Arabidopsis *etr2-1* (**Supplementary Fig. S2**; Sakai et al. 1998). This proline residue in the first domain is highly conserved amongst known and/or putative ethylene-binding proteins (Wang et al. 2006). *Nr* is reported to show an ethylene-insensitive phenotype in the seedling triple response assay. In the dose response assay of exogenously applied ethylene, *Nr* responded to a low concentration of ethylene (1 p.p.m.) (Lanahan et al. 1994). In contrast, *Sletr1-1* showed no response to 10 p.p.m. ethylene, indicating that this mutant is less sensitive to ethylene than is *Nr*. The ethylene insensitivity of *Sletr1-1* resembled that of Arabidopsis *etr1-1* (*Atetr1-1*), which is completely insensitive to ethylene. The relationship between amino acid substitution and ethylene binding capacity was investigated in Arabidopsis, and it was found that the binding capacity of P36L-mutated AtETR1 was <5% that of the wild-type protein (Wang et al. 2006). This result supports the hypothesis that P51L-mutated SlETR1 is incapable of binding to ethylene. The second affected allele, V69, was located near the ethylene-binding domain, where the effect on ethylene binding was previously reported (Rodriguez et al. 1999, Wang et al. 2006). The hydrophobicity of the transmembrane region appears to be an important determinant of the membrane orientation (Harley et al. 1998). The calculated hydrophobicity was reduced as a result of the V69D substitution in the Sletr1-2 protein. However, no experimental evidence has been reported to date that demonstrates the importance of this residue in ethylene binding activity. The fact that *Sletr1-2* exhibited an ethylene-insensitive phenotype suggests that the V69D substitution of *Sletr1-2* may also affect ethylene binding activity.

Homozygous *Sletr1-1* and *Nr* showed similar fruit ripening phenotypes, both being impaired in ripening at 60 DAF (**Fig 5A**). The ripening phenotypes of the heterozygous lines differed from those of the homozygous lines. Whereas the fruit of heterozygous *Sletr1-1* finally turned red (**Fig. 5C**), the ripening of *Nr* fruit remained incomplete with respect to color (Lanahan et al. 1994). Since we were concerned about the effect of the different genetic backgrounds of *Sletr1* (Micro-Tom) and *Nr* (Ailsa Craig), we evaluated the petal abscission phenotype in *Nr* (*Nr*/*Nr*) (cv. Rutgers)×wild type (*nr*/*nr*) (cv. Micro-Tom) BC_1 plants (*Nr*-MT BC_1). We used *Nr* in the Rutgers background for this experiment, since *Nr* in the Ailsa Craig background was unavailable at the time. Heterozygous *Sletr1-1* exhibited impaired petal abscission, whereas the effect was weaker for *Nr*-MT BC_1 (data not shown), indicating that the ripening and petal abscission phenotypes of heterozygous

Sletr1-1 were opposite to those of *Nr*-MT BC_1. To test if we could obtain a similar result with *Sletr1-1* in a different genetic background, we generated F_1 heterozygous lines of *Sletr1-1* crossed with Ailsa Craig. These lines bore red fruits and exhibited an apparent delay in petal abscission (data not shown). Whereas *Sletr1-1* shows a stronger degree of ethylene insensitivity than *Nr* in the seedling, the opposite is true during the fruit ripening process. *NR* gene expression is up-regulated at the breaker stage together with the initiation of autocatalytic ethylene biosynthesis at the onset of ripening, whereas *SlETR1* gene expression stayed at a constant level during fruit development (Lashbrook et al. 1998). The gene expression pattern of *SlETR1* and *NR* during fruit development (Lashbrook et al. 1998) also supports this idea that *NR* is important in the fruit ripening process. Together with previous reports, our data suggest that *SlETR1* and *Nr* are functionally distinct in petal abscission and fruit ripening. Although no clear ripening phenotype was observed for *Sletr1-2* (**Fig. 5A**), deterioration of postharvest fruit was prolonged compared with that in the wild type (**Fig. 6**), indicating that the weak ethylene insensitivity affected an aspect of fruit ripening. Improvement of postharvest fruit shelf life is an important agronomic trait in tomato breeding. Modifying the key regulatory factors of fruit ripening and softening may prolong shelf life and provides massive commercial and economical benefit. Ethylene controls the fruit ripening process of climacteric fruits via the ethylene biosynthetic and signaling pathway (Barry and Giovannoni 2007). While several ripening mutants, such as *rin*, *nor* and *Nr*, have been identified (Barry and Giovannoni 2007), only *rin* has been used in practical breeding applications (Kitagawa et al. 2005). Although *Nr* produces fruit with a good shelf life, it has not been used in breeding programs due to its incomplete ripening phenotype even in the heterozygous state (*Nr*/*nr*), which results in insufficient red coloration (Lanahan et al. 1994). Furthermore, there are traits associated with *Nr* that are undesirable, such as increased susceptibility to some pathogens (Francia et al. 2007, Kavroulakis et al. 2007, Cantu et al. 2009). Mutants with drastically altered phenotypes may be useful material for basic studies, but are often not suitable for use in breeding. In this regard, since *Nr* has a more pronounced impairment of ripening than does *Sletr1*, the *Sletr1* alleles are more suitable for use in breeding programs. The identification of two novel *Sletr1* mutant alleles that are distinguished by the level of ethylene sensitivity and the characterization of their associated phenotypes could provide insight into the ethylene-mediated fruit ripening mechanism in tomato.

Materials and Methods

Plant material

Tomato (*S. lycopersicum*) cv. Micro-Tom was used. The TILLING population was developed as previously described (Watanabe et al. 2007, Saito et al. 2011). EMS-mutagenized plants were grown in a greenhouse.

DNA extraction and pooling

For each M_2 line, genomic DNA was isolated from 10 individual plants. Leaves were collected in 2 ml micro test tubes, frozen in liquid nitrogen, and homogenized with a pestle. Genomic DNA was extracted using a Maxwell16 Tissue DNA Purification Kit (Promega). To generate DNA superpools in 96-well format, DNA from eight lines was mixed in one well. The concentration of DNA was normalized by separating DNA samples on an agarose gel by electrophoresis and comparing the intensity of the ethidium bromide-stained bands with that of a λDNA reference (New England Biolabs).

PCR amplification

PCR primers were designed using the PRIMER3 program (Rozen et al. 2000) after selecting an optimal region to screen using the CODDLE program (Till et al. 2003) except for *SlSSADH* and *SlGABAT1* for which entire coding regions were targeted. PCR amplification was carried out by two methods, either nested PCR was performed using universal primers or gene-specific primers were used. In the gene-specific primer system, PCR was carried out by direct PCR using IRD700 and IRD800-labeled primers; in the universal primer system, non-labeled gene-specific primers attached to the T7 (CGCGTAATACGACTCACTATAG) or SP6 (CATACGATTTAGGTGACACTATAG) sequence at the 5′ end were used in the first round of PCR. PCRs were performed in a total volume of 15 μl, with 5–10 ng of superpool DNA. DNA was mixed with 1.5 μl of 5× Green Gotaq Flexi buffer, 2.0 μl of 25 mM $MgCl_2$, 1.2 μl of 2.5 mM dNTP, 0.3 μl of 10 μM primers and 0.075 μl of 5 U μl^{-1} Gotaq Hotstart DNA polymerase or Gotaq Flexi DNA Polymerase (Promega). Then, 0.5 μl from the first PCR was used as a template in the second nested PCR using IRD700- and IRD800-labeled T7 and SP6 primers (biomers.net). After confirming successful PCR amplification by electrophoresis, 3–7 μl of PCR products was used in the subsequent TILLING screening.

Preparation of tomato ENDO1 nuclease and detection of mismatch DNA

Coding sequences of SlENDO1 (accession No. AB667996) were cloned into the pGWB8 binary vector (Nakagawa et al. 2007). Recombinant SlENDO1 tagged with six histidine residues was stably expressed in tomato cv. Micro-Tom. SlENDO1 protein was extracted from transgenic tomato leaves. We basically followed a previously described protein purification method (Triques et al. 2007), with some modifications. A 2 g aliquot of tomato leaves was homogenized with a mortar and pestle in 10 ml of buffer [0.1 M Tris–HCl (pH 8.0), 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.125 mM 2-mercaptoethanol and 10% glycerol]. The extract was cleared by centrifugation at 16,000×*g* for 20 min. The supernatant was used for further purification. Recombinant SlENDO1 was purified using a Histrap HP Column (GE Healthcare). The eluted fraction was dialyzed as previously described (Triques et al. 2007). The

enzyme activity for each batch of SlENDO1 was determined using a dilution series of control samples (**Supplementary Fig. S5B**). PCR products were mixed with sterilized water up to a total volume of 10 µl and subjected to SlENDO1 digestion as previously described (Triques et al. 2007). TILLING screening was performed using an LI-COR DNA analyzer as previously described (Till et al. 2006) and as shown in **Fig. 1**.

Selection of TILLING mutant alleles in bulked M_3 line populations

To select mutant individuals, a CAPS marker was designed for each identified allele to distinguish the mutant allele from that of the wild type. Appropriate restriction enzymes were selected from those listed by the PARSESNP tool (Taylor and Greene. 2003), as shown in **Supplementary Fig. S3**. If no restriction site was found at the position of mutation, the target region was amplified with TILLING primers used in the screening, and the obtained PCR products (400–500 ng) were digested with SlENDO1. Digested fragments were visualized by electrophoresing in a 1.5–2.0% standard agarose gel followed by SYBR Safe DNA gel staining (Invitrogen).

Seedling triple response assay and epinastic response assay

Seeds were sterilized with 10% commercial bleach including a detergent (Kitchen Haiter, Kao) for 20 min and then rinsed with sterilized water three times for 5 min each. The seeds were germinated in a 50 ml glass bottle containing 10 ml of 1/2 MS medium (Murashige and Skoog 1962). Ethylene was added to the bottles sealed with silicon rubber at the designed concentrations (5 or 10 p.p.m.) and seedlings were grown for 7 d in the dark at 25°C. The epinasitic response assay was performed using 4-week-old plants, ethylene was injected into the sealed chambers at the designed concentration (50 p.p.m.), and the plants were incubated in growth chambers for 20 h in the light at 25°C.

Characterization of mutant phenotypes

Seeds of wild-type Micro-Tom and BC_1S_1 and BC_1S_2 homozygous seeds of *Sletr1-1* and *Sletr1-2* were sown on wet filter paper and placed under continuous light for 2–3 d at 25°C to stimulate seed germination. Germinated seeds were then transplanted into soil and grown in a photoperiod of 16 h light at 25°C. The date of flowering (0 DAF) was tagged for the time course observation of flower abscission, fruit ripening and evaluation of fruit shelf life at the times indicated in **Figs. 5** and **6**. All plants were grown under the same conditions as described above.

Genotyping of *Sletr1* mutant alleles

Homozygous and heterozygous *Sletr1* plants were selected from BC_1S_1 populations as follows. CAPS analysis was used to distinguish the *Sletr1-1* or *Sletr1-2* allele from the wild-type *SlETR1* allele. PCR amplification for the detection of each mutation was performed with a SlETR1-CAPS forward primer (5′-gtataaaagg agttggggcaaag-3′) and SlETR1-CAPS reverse primer (5′-atcagg aatgatgtggacaagc-3′) to yield a 730 bp PCR product. PCR products were digested with *Xsp*I (TAKARA) and *Mbo*I (New England Biolabs) to detect *Sletr1-1* and *Sletr1-2* alleles, respectively. The *Xsp*I-digested *Sletr1-1* product consisted of a 503 and 227 bp fragment. *Xsp*I digestion of *SlETR1/Sletr1-1* heterozygous plants resulted in three fragments, the 730 bp fragment (non-digested wild-type allele) and a 503 and 227 bp fragment (digested *Sletr1-1* allele). *Mbo*I-digested *Sletr1-2* consisted of a 159, 397 and 174 bp fragment. *Mbo*I digestion of *SlETR1/Sletr1-2* heterozygous plants resulted in four fragments, of 159 and 571 bp (partially digested wild-type allele) and of 397 and 174 bp (digested *Sletr1-2* allele).

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